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(54) **Improved variant of D-psicose 3-epimerase and uses thereof**

(57) The present invention relates to an improved variant of a D-psicose 3-epimerase and its uses.

EP 2 843 044 A1

Description

FIELD OF THE INVENTION

[0001] The present invention relates to an improved isomerase, in particular D-psicose-3-epimerase, for preparing psicose from fructose and its uses.

BACKGROUND OF THE INVENTION

[0002] D-psicose, also called D-allulose, is a rare sugar isomer of fructose. It can be found in nature but at very low concentrations like in edible mushrooms or the jackfruit, in wheat and the *Itea* plants.

[0003] At the opposite of fructose, the metabolism of psicose in humans is partly absorbed and metabolized in energy, and partly excreted unchanged in the urine and in the faeces.

[0004] The characteristics of D-psicose as a material for preventing lifestyle-related diseases have been disclosed, including its noncaloric nature, a positive effect on the reduction of the glycemic response, an antiobesity effect, and the like. In addition, the sweetness of D-psicose is about 70 % of that of sucrose (Oshima, et al. (2006) Psicose contents in various food products and its origin. Food Sci Technol Res 12:137-143), but 0.3% energy of sucrose and is suggested as an ideal sucrose substitute for food products. It can also be used as an inhibitor of hepatic lipogenic enzyme and intestinal α-glycosidase for reducing body fat accumulation. It further shows important physiological functions, such as reactive oxygen species scavenging activity and neuroprotective effect. In addition, it also improves the gelling behavior and produces good flavor during food process.

[0005] D-psicose exists in extremely small quantities in commercial carbohydrate or agricultural products and is difficult to chemically synthesize. Therefore, interconversion between D-fructose and D-psicose by epimerization using D-tagatose 3-epimerase (DTEase) family enzymes has been confused on as attractive way of D-psicose production.

[0006] So far, there have been 9 kinds of DTEase family enzyme sources reported. Twenty years ago, DTEase was firstly characterized by Izumori et al. from *Pseudomonas cichorii,* showing C-3 epimerization activity of ketohexoses with the optimum substrate of D-tagatose (Izumori et al. 1993, Biosci. Biotechnol. Biochem. 57, 1037-1039). Till 2006, the second enzyme with C-3 epimerization activity of ketohexoses was identified from *Agrobacterium tumefaciens,* and it was named D-psicose 3-epimerase (DPEase), due to its high substrate specificity for D-psicose (Kim et al. 2006, Applied and environmental microbiology 72, 981-985; US 2010/0190225, WO2011/040708). Recently, another six DTEase family enzymes were characterized from *Rhodobacter sphaeroides* SK011 (DTEase) (Zhang et al. 2009, Biotechnology letters 31, 857-862), *Clostridium cellulolyticum* H10 (DPEase) (Mu et al. 2011, Journal of agricultural and food chemistry 59, 7785-7792, CN102373230), *Ruminococcus* sp. 5_1_39BFAA (DPEase) (Zhu et al. 2012, Biotechnology letters 34, 1901-1906), *Clostridium bolteae* ATCC BAA-613 (Jia et al. 2013, Applied Microbiology and Biotechnology DOI 10.1007/s00253-013-4924-8), *Clostridium scindens* ATCC 35704 (Zhang et al. 2013, PLoS ONE 8, e62987), and *Clostridium* sp. BNL1100 (Mu et al. 2013, Biotechnology Letter DOI 10.1007/s10529-013-1230-6), respectively. In addition, Maruta et al. disclosed a DTEase producing source in *Rhizobium* (US 2011/0275138).

[0007] There is only one reference to report the enzyme modification of DTEase family enzymes by protein engineering technology. Using random and site-directed mutagenesis technology, Choi et al. (2011, Applied and environmental microbiology 77, 7316-7320) constructed the I33L S213C double-site variant of *A. tumefaciens* DPEase, and the variant enzyme showed increases in optimal temperature, half-life, melting temperature, and the catalysis efficiency, compared with the wild-type enzyme. Its optimal pH remains unchanged at 8.00.

[0008] However, the enzymes have optimum pH for activity at 8.0 - 9.5, and the pH stability is between 8.0 - 10.0, which is not appropriate for industrial application.

[0009] Therefore, the main concern for using psicose remains its scarcity and its production cost and the need for improved industrial D-psicose production still exists.

SUMMARY OF THE INVENTION

[0010] To develop industrial D-psicose production and reduce the production cost, an optimized DTEase family enzyme should be weak-acid stable and thermostable, and has a higher catalysis efficiency and turnover for the substrate D-fructose.

[0011] The present invention relates to a variant of a parent D-psicose 3-epimerase, wherein the variant comprises a substitution of a glycine residue by a serine residue at a position corresponding to the position 211 in SEQ ID No 2 compared to the parent D-psicose 3-epimerase; and wherein the variant has a D-psicose 3-epimerase activity.

[0012] In a preferred embodiment, the variant has one or several following features:

a. a lower pH optimum compared to the parent D-psicose 3-epimerase, preferably in the range of 6 to 7; and/or

b. a higher catalysis efficiency to the substrate D-fructose compared to the parent-psicose 3-epimerase, preferably at least twice higher; and/or
c. a longer half-life at 60°C compared to the parent-psicose 3-epimerase.

[0013] Preferably, the variant has an amino acid sequence having 35 % of identity or higher with SEQ ID No 2, preferably 60 % of identity or higher, more preferably at least 70, 75, 80, 85, 90, 95 % of identity or higher.

[0014] In a preferred embodiment, the parent D-psicose 3-epimerase is selected from a D-tagatose 3-epimerase from *Pseudomonas cichorii,* a D-psicose 3-epimerase from *Agrobacterium tumefaciens,* a D-psicose 3-epimerase from *Clostridium sp,* a D-psicose 3-epimerase from *Clostridium scindens,* a D-psicose 3-epimerase from *Clostridium bolteae,* a D-psicose 3-epimerase from *Ruminococcus sp,* and a D-psicose 3-epimerase from *Clostridium cellulolyticum.* More preferably, the parent D-psicose 3-epimerase is the D-psicose 3-epimerase from *Clostridium cellulolyticum.* In a most preferred embodiment, the variant comprises or consists of the amino acid sequence of SEQ ID No 4 or an amino acid sequence having 90 or 95 % of identity with SEQ ID No 4 or higher and having a residue serine at position 211.

[0015] Another object of the present invention is an isolated nucleic acid encoding a variant according to the present invention. The present invention further relates to an expression cassette or recombinant expression vector comprising a nucleic acid encoding a variant according to the present invention. It also relates to a recombinant host cell comprising a nucleic acid according to the present invention, an expression cassette according to the present invention or a recombinant expression vector according to the present invention. In a particular embodiment, the host cell is a GRAS strain (Generally Recognized As Safe), preferably *Bacillus subtilis.*

[0016] The present invention relates to a method for producing a D-psicose 3-epimerase variant comprising culturing the recombinant host cell according to the present invention, and optionally recovering or purifying the produced D-psicose 3-epimerase variant from the resulting culture. In other word, it relates to the use of a recombinant host cell according to the present invention for producing a D-psicose 3-epimerase variant according to the present invention.

[0017] The present invention also relates to a method for producing D-psicose comprising contacting a variant according to the present invention with D-fructose in conditions suitable for the D-psicose 3-epimerase activity and optionally recovering the produced D-psicose. Optionally, the D-fructose is previously or simultaneously produced by a glucose isomerase from D-glucose. Then, the present invention relates to the use of a D-psicose 3-epimerase variant according to the present invention or a recombinant host cell according to the present invention for producing D-psicose.

[0018] An object of the present invention is an enzymatic composition comprising a D-psicose 3-epimerase variant according to the present invention and an additional enzyme, in particular a glucose isomerase.

[0019] Finally, the present invention relates to the use of a GRAS host cell according to the present invention for preparing a food product and to a food product comprising such a GRAS host cell.

DETAILED DESCRIPTION OF THE INVENTION

[0020] The present invention relates to an improved variant of a D-psicose 3-epimerase.

Definitions

[0021] In the present document, the term "DPEase" and "DTEase" could be used in place of "D-psicose 3-epimerase" and "D-tagatose 3-epimerase", respectively. And "DPEase" and "DTEase" mean the ketose 3-epimerases with the optimum substrates as D-psicose and D-tagatose, respectively. In addition, the term "DPEase variant" may also referred to a variant of a D-tagatose 3-epimerase as taught in the present invention.

[0022] Identity Percentage: The "percentage identity" between two amino acid sequences (A) and (B) is determined by comparing the two sequences aligned in an optimal manner, through a window of comparison. Said alignment of sequences can be carried out by well-known methods, for example, using the algorithm for global alignment of Needleman-Wunsch. Protein analysis software matches similar sequences using measures of similarity assigned to various substitutions, deletions and other modifications, including conservative amino acid substitutions. Once the total alignment is obtained, the percentage of identity can be obtained by dividing the full number of identical amino acid residues aligned by the full number of residues contained in the longest sequence between the sequence (A) and (B). Sequence identity is typically determined using sequence analysis software. For comparing two amino acid sequences, one can use, for example, the tool "Emboss needle" for pairwise sequence alignment of proteins providing by EMBL-EBI and available on www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=emboss_needle&context=protein, using default settings : (I) Matrix : BLOSUM62, (ii) Gap open : 10, (iii) gap extend : 0.5, (iv) output format : pair, (v) end gap penalty : false, (vi) end gap open : 10, (vii) end gap extend : 0.5.

[0023] By "about" is intended the value more or less 10 % of the value. Preferably, it is intended the value more or less 5 % of the value. For instance, "about 100" means between 90 and 110, preferably between 95 and 105.

[0024] By "D-psicose 3-epimerase activity" is referred the capacity of the enzyme to modify D-fructose into D-psicose.

This activity can be assayed by measuring the amount of D-psicose formed from D-fructose. In particular, it can be measured as detailed in the Example section or as disclosed in Mu et al. (2011, Journal of agricultural and food chemistry 59, 7785-7792; in "Enzyme Assay" section page 7787).

Variant of D-psicose 3-epimerase

[0025] The present invention relates to a variant of a parent D-psicose 3-epimerase, wherein the variant comprises a substitution of a glycine residue by a serine residue at a position corresponding to the position 211 in SEQ ID No 2 compared to the parent D-psicose 3-epimerase; and wherein the variant has a D-psicose 3-epimerase activity.

[0026] The inventors surprisingly identified a G211S variant of DPEase from *C. cellulolyticum* as an improved variant. Indeed, this variant presents the following advantages (see Tables 1 and 2):

- a lower pH optimum, namely 6.5 instead of 8.0 for the wild-type DPEase;
- a higher half-life at 60°C, namely 7.2 h instead of 6.8; and
- a higher k_{cat}/K_m for D-fructose, namely 150.6 instead of 62.7.

[0027] According to the knowledge of the inventors, it is the first time that a DPEase is reported with a pH optimum lower than 7.0. In addition, the lowering of the pH optimum goes along with an improved stability and a strong increase of catalytic efficiency.

[0028] Accordingly, the DPEase variant has one or several following features:

a. a lower pH optimum compared to the parent D-psicose 3-epimerase, preferably in the range of 6 to 7; and/or
b. a higher catalysis efficiency to the substrate D-fructose compared to the parent-psicose 3-epimerase, preferably at least 50, 75, 100, 120 % higher, more preferably at least twice higher; and/or
c. a longer half-life at 60°C, preferably of at least 5, 10, 15 or 20 minutes longer.

[0029] In a first embodiment, the DPEase variant fulfils the requirement of items a) and b), items a) and c), items b) and c), or items a), b) and c). Preferably, the DPEase variant has a lower pH optimum compared to the parent D-psicose 3-epimerase, preferably in the range of 6 to 7. Therefore, the DPEase variant fulfils the requirement of items a) and b), items a) and c), or items a), b) and c).

[0030] The inventors further noted that, despite a quite low amino acid (aa) sequence identity between D-tagatose 3-epimerase from *Pseudomonas cichorii,* D-psicose 3-epimerase from *Agrobacterium tumefaciens,* and D-psicose 3-epimerase from *Clostridium cellulolyticum* (i.e., DTEase of *P. cichorii* has 41 % aa identity with DPEase of *C. cellulolyticum;* DPEase of *A. tumefaciens* has 60 % aa identity with DPEase of *C. cellulolyticum*), the residue G211 of the DPEase of *C. cellulolyticum* is conserved. Furthermore, as shown in Figure 1, G211 is conserved seven of the eight enzymes (see Figure 1).

[0031] Then, the present invention relates to a DPEase variant having an amino acid sequence having 35 % of identity or higher with SEQ ID No 2, preferably 60 % of identity or higher, more preferably at least 70, 75, 80, 85, 90, 95 % of identity or higher. It is obviously understood that all the DPEase variants of the present invention present the substitution of Gly by Ser at the position corresponding to the residue 211 in SEQ ID No 2.

[0032] More particularly, the parent D-psicose 3-epimerase is selected from a D-tagatose 3-epimerase from *Pseudomonas cichorii,* a D-psicose 3-epimerase from *Agrobacterium tumefaciens,* a D-psicose 3-epimerase from *Clostridium sp,* a D-psicose 3-epimerase from *Clostridium scindens,* a D-psicose 3-epimerase from *Clostridium bolteae,* a D-psicose 3-epimerase from *Ruminococcus sp,* and a D-psicose 3-epimerase from *Clostridium cellulolyticum.* In a preferred embodiment, the parent D-psicose 3-epimerase is a D-psicose 3-epimerase from *Clostridium cellulolyticum,* more particularly *Clostridium cellulolyticum* strain H10 (ATCC 35319).

[0033] Therefore, the present invention relates to a DPEase variant having or comprising the amino acid sequence of SE ID No 2 with a G211S substitution (i.e., the amino acid sequence of SEQ ID No 4) or an amino acid sequence having 90 or 95 % of identity with SEQ ID No 4 or higher and having a residue Ser at position 211.

[0034] Alternatively, it also relates to a DPEase variant having or comprising the amino acid sequence of SE ID No 5 with a G211S substitution or an amino acid sequence having 90 or 95 % of identity with SEQ ID No 5 or higher and having a residue Ser at position 211.

[0035] In addition, it also relates to a DPEase variant having or comprising the amino acid sequence of SE ID No 6 with a G210S substitution or an amino acid sequence having 90 or 95 % of identity with SEQ ID No 6 or higher and having a residue Ser at position 210.

[0036] Alternatively, it also relates to a DPEase variant having or comprising the amino acid sequence of SE ID No 7 with a G211S substitution or an amino acid sequence having 90 or 95 % of identity with SEQ ID No 7 or higher and having a residue Ser at position 211.

[0037] In addition, it also relates to a DPEase variant having or comprising the amino acid sequence of SE ID No 8 with a G213S substitution or an amino acid sequence having 90 or 95 % of identity with SEQ ID No 8 or higher and having a residue Ser at position 213.

[0038] Alternatively, it also relates to a DPEase variant having or comprising the amino acid sequence of SE ID No 9 with a G223S substitution or an amino acid sequence having 90 or 95 % of identity with SEQ ID No 7 or higher and having a residue Ser at position 223.

[0039] Finally, it also relates to a DTEase variant having or comprising the amino acid sequence of SE ID No 10 with a G213S substitution or an amino acid sequence having 90 or 95 % of identity with SEQ ID No 10 or higher and having a residue Ser at position 213.

[0040] Optionally, the variant has alterations at not more than 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acids, e.g., may have substitution, insertion, and/or deletion of 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids.

[0041] The present invention also relates to a DPEase variant according to the present invention further comprising a tag. For instance, it can comprise tag suitable for facilitate the DPEase variant purification or immobilization, such as a His tag (His_6), a FLAG tag, a HA tag (epitope derived from the Influenza protein haemagglutinin), a MYC tag (epitope derived from the human proto-oncoprotein MYC) or a GST tag (small glutathione-S-transferase).

[0042] Finally, the present invention relates to a DPEase variant according to the present invention immobilized on a solid support or a carrier. The DPEase can be immobilized on any suitable support or carrier, such as alginate, amberlite resin, Sephadex resin or Duolite resin, e.g., beads. Immobilization means are well-know to the person skilled in the art. For instance, see Choi et al, *supra*; Lim et al. (2009, Porcess Biochemistry 44, 822-828) and WO2011/040708, the disclosure thereof being incorporated herein by reference.

Nucleic acid, vector and host cells

[0043] The present invention relates to a nucleic acid encoding a DPEase variant according to the present invention or a nucleic acid comprising a sequence encoding a DPEase variant according to the present invention. The present invention also relates to an expression cassette of a nucleic acid according to the invention. It further relates to a vector comprising a nucleic acid or an expression cassette according to the invention. Preferably, the vector is an expression vector. The vector is preferably a plasmid vector. In addition, the present invention relates to a host cell comprising a nucleic acid according to the invention, an expression cassette of a nucleic acid according to the invention or a vector comprising a nucleic acid or an expression cassette according to the invention. The nucleic acid encoding a DPEase variant according to the present invention can be present in the host cell as an episomic sequence or can be incorporated into its chromosome. The nucleic acid encoding a DPEase variant according to the present invention can be present in the host cell in one copy or in several copies.

[0044] The nucleic acid can be DNA (cDNA or gDNA), RNA, or a mixture of the two. It can be in single stranded form or in duplex form or a mixture of the two. It can comprise modified nucleotides, comprising for example a modified bond, a modified purine or pyrimidine base, or a modified sugar. It can be prepared by any method known to one skilled in the art, including chemical synthesis, recombination, mutagenesis etc...

[0045] The expression cassette comprises all elements required for expression of the DPEase variant according to the present invention, in particular the elements required for transcription and translation in the host cell, in particular in the considered host cell.

[0046] The host cell can be prokaryotic or eukaryotic, preferably prokaryotic or lower eukaryotic, more preferably prokaryotic. In particular, the expression cassette comprises a promoter and a terminator, optionally an enhancer. The promoter can be prokaryotic or eukaryotic, depending on the selected host cell. Examples of preferred prokaryotic promoters include: LacI, LacZ, pLacT, ptac, pARA, pBAD, the RNA polymerase promoters of bacteriophage T3 or T7, the polyhedrin promoter, the PR or PL promoter of lambda phage. In general, to select a suitable promoter, one skilled in the art may advantageously consult Sambrook et al. work (1989) or techniques described by Fuller et al. (1996; Immunology in Current Protocols in Molecular Biology).

[0047] The present invention relates to a vector containing a nucleic acid or an expression cassette encoding the DPEase variant according to the present invention. The vector is preferably an expression vector, that is to say, it comprises the elements required for the expression of the variant in the host cell. The vector is a self-replicable vector. The host cell can be a prokaryote, for example *E. coli,* or a eukaryote. The eukaryote can be a lower eukaryote such as a yeast (for example, *S. cerevisiae*) or fungus (for example from the genus *Aspergillus* or *Actinomyces*) or a higher eukaryote such as an insect, mammalian or plant cell. The cell can be a mammalian cell, for example COS, CHO (US 4,889,803; US 5,047,335). In a particular embodiment, the cell is non-human and non-embryonic.

[0048] The vector can be a plasmid, phage, phagemid, cosmid, virus, YAC, BAC, pTi plasmid from *Agrobacterium,* etc... The vector can preferably comprise one or more elements selected from the group consisting of a replication origin, a multiple cloning site and a selection gene. In a preferred embodiment, the vector is a plasmid. The vector is a self-replicable vector. Examples of prokaryotic vectors include, but are not limited to, the following: pER322, pQE70, pMA5,

pUC18, pQE60, pUB110, pQE-9 (Qiagen), pbs, pTZ4, pC194, pD10, pHV14, Yep7, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16A, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pBR322, and pRIT5 (Pharmacia), pET (Novagen). Examples of eukaryotic vectors include, but are not limited to, the following: pWL-NEO, pSV2CAT, pPICZ, pcDNA3.1 (+) Hyg (Invitrogen), pOG44, pXT1, pSG (Stratagene); pSVK3, pBPV, pCI-neo (Stratagene), pMSG, pSVL (Pharmacia); and pQE-30 (QLAexpress). Preferably the expression vector is a plasmid vector.

[0049] More particularly, to express in *E. coli,* pBR322, pUC18, pBluescript II SK (+), λgt.λC and λgt.λB can be preferably used. While, to express in *Bacillus subtilis,* pUB110, pTZ4, pC194, p11, Φ1 and Φ105 can be preferably used. Plasmids, pHV14, TRp7, YEp7 and pBS7 are useful in the case of replicating the recombinant nucleic acid in two or more kinds of hosts. In order to insert encoding nucleic acid sequence into these vectors, conventional methods in the art can be used.

[0050] In a particular embodiment, the vector is an integration vector suitable to incorporate the sequence encoding the DPEase variant according to the present invention into the chromosome of the host cell. A non-exhaustive example of commercially available integration vectors is pMUTIN4 for *B. substilis* from Bacillus Genetic Stock Center.

[0051] The host cell can be preferably selected among the group consisting of *E. coli,* and GRAS strains. Preferably, the GRAS strain is selected from the group consisting of innocuous bacteria, especially innocuous *Corynebacterium sp.* such as *C. glutamicum,* and innocuous *Bacillus sp.* such as *B. subtilis.* In a very specific embodiment, the host cell is of *E. coli* or *B. subtilis,* preferably *B. Subtilis.*

[0052] The present invention relates to the use of a nucleic acid, an expression cassette, an expression vector or a host cell as disclosed above for producing a DPEase variant according to the present invention.

[0053] It also relates to a method for producing a DPEase variant according to the present invention comprising culturing the recombinant host cell according to the present invention, and optionally recovering and/or purifying the produced D-psicose 3-epimerase variant from the resulting culture. In a preferred embodiment, the host cell is selected from *E. coli,* and GRAS strains, especially *B. subtilis.*

[0054] Optionally, the host cell further produces a glucose isomerase.

[0055] In a particular embodiment, the present invention relates to an immobilized host cell according to the present invention producing and secreting a DPEase variant of the present invention.

Production of D-psicose

[0056] The present invention relates to the use of a DPEase variant according the present invention for producing D-psicose and to the method for producing D-psicose by using a DPEase variant according the present invention.

[0057] In a first embodiment, the DPEase variant is contacted with D-fructose in conditions suitable for the D-psicose 3-epimerase activity. D-fructose can be provided as high fructose syrup, and in particular high fructose corn syrup. Such high fructose corn syrups are commercially available from Roquette Freres under HI-SWEET® references.

[0058] In another particular embodiment, D-glucose is contacted with an enzyme mixture comprising a DPEase variant according the present invention and a glucose isomerase. The glucose isomerase is also called xylose isomerase and corresponds to EC. 5.3.1.5. Preferably, glucose is provided as a glucose syrup, in particular a corn syrup.

[0059] In another alternative, the starting material may be starch in place of glucose or fructose, and the enzyme mixture further comprises alpha-amylase and/or glucoamylase.

[0060] The present invention relates to an enzyme mix comprising a DPEase variant according the present invention and an additional enzyme. Preferably, the enzyme mix comprises a DPEase variant according the present invention and a glucose isomerase. Optionally, the enzyme mix may further comprise alpha-amylase and/or glucoamylase.

[0061] Suitable conditions for producing D-psicose can be defined by the person skilled in the art. Preferably, they include the following features:

- Temperature: between 50 and 60°C, preferably about 55°C; and/or
- pH: between 5.5 and 7.5, preferably between 6 and 7, more preferably about 6.5; and/or
- in presence of a divalent metal ion, preferably selected from the group consisting of Co^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+} and Mg^{2+}, more preferably from Co^{2+}, Mn^{2+}, Fe^{2+}, and Ni^{2+}, still preferably from Co^{2+} and Mn^{2+}, and most preferably Co^{2+}; and/or
- in presence of borate when immobilized TDEase is used, e.g., 40-80 mM of borate, preferably about 60 mM .

[0062] In a particular embodiment, the enzymes to be used in the method are immobilized. More particularly, the DPEase variant of the present invention is immobilized. Optionally, both glucose isomerase and DPEase variant of the present invention can be immobilized or solely the DPEase variant. In another alternative, instead of immobilizing the enzyme, the microorganisms producing the enzymes are immobilized. The enzyme(s) or microorganisms can be for instance immobilized on any suitable support, such as alginate, amberlite resin, Sephadex resin or Duolite resin, e.g., beads.

[0063] The immobilized enzyme(s) or microorganisms can be packed into a suitable column and the glucose or fructose liquid or syrup is continuously introduced into the column.

[0064] Methods for immobilized DPEases on a support and to produce D-psicose are well known to the person skilled in the art, for instance in WO2011/040708.

[0065] The resulting product can be a mixture of D-fructose and D-psicose, and even a mixture of D-glucose, D-fructose and D-psicose.

[0066] An aspect of the present invention relates to the use of a GRAS host cell according to the present invention for preparing a food product and to a food product comprising such a GRAS host cell. The food products are for humans or for animal feed. For instance, foods can be foods for health, foods for patients, food materials, food materials for health, food materials for patients, food additives, food additives for health, food additives for patients, beverages, beverages for health, beverages for patients, potable water, potable water for health, potable water for patients, drugs, drug raw materials, feeds, and feeds for diseased domestic animals and/or diseased animals. When used as a food material or a food additive, it can be used for alleviating abnormal carbohydrate metabolism and/or abnormal lipid metabolism. It may be in the form of a solid preparation such as a tablet, a capsule, or a powder or granules to be dissolved in beverages, etc.; a semisolid preparation such as jelly; a liquid such as potable water; a high-concentration solution to be diluted before use; or the like.

BRIEF DESCRIPTION OF THE DRAWINGS

[0067]

Figure 1. Multiple sequence alignment of DTEase family enzymes and their homologs. Amino acid sequence were from *C. cellulolyticum* DPEase (Clce-DPEase; GeneBank accession No: ACL75304), *Clostridium* sp. (Clsp-DPEase; YP_005149214.1), *C. scindens* DPEase (Clsc-DPEase; EDS06411.1), *A. tumefaciens* DPEase (Agtu-DPEase; AAL45544), *C. bolteae* DPEase (Clbo-DPEase; EDP19602), *Ruminococcus* sp. DPEase (Rusp-DPEase; ZP_04858451), *P. cichorii* DTEase (Psci-DTEase; BAA24429), and *R. sphaeroides* DTEase (Rhsp-DTEase; AC059490). The alignment was performed using ClustalW2 program (www.ebi.ac.uk/Tools/clustalw2/index.html). Amino acid residues that are identical in all the displayed sequences are marked by asterisks (*), strongly conserved or weakly conserved residues are indicated by colons (:) or dots (.), respectively.

Figure 2. Comparison of pH profiles between wild-type and G211S mutant of *C. cellulolyticum* DPEase.

Figure 3. SDS-PAGE analysis of purified G211S DTEase variant and protein markers stained with Coomassie blue. Lane 1, *Bacillus subtilis* producing DPEase; Lane 2, protein marker.

EXAMPLES

[0068] The inventors prepared, by site-directed mutagenesis, DPEase variants of *C. cellulolyticum* by replacing the codon GGC encoding Gly in position 211 (SEQ ID No 1) by the codons AGC, GCC, GAC, CGC, TGG and CTC, encoding respectively the substitutions G211 S, G211A, G211D, G211T, G211W and G211L.

[0069] The DPEase variants have been expressed in *Bacillus subtilis,* expressed and purified (Figure 3).

[0070] Enzyme properties and kinetic parameters of the wild-type and variants of DPEase from C. *cellulolyticum* for substrate D-psicose have been determined and the results are given in Table 1.

[0071] The G211S variant showed improved characteristics in comparison with the wildtype DPEase (Table 1), but also with the other known enzymes (Table 2). In particular, it is a weak-acid stable enzyme with more than 80 % activity in the pH range from 6 to 8 (Figure 2), has a catalysis efficiency of approximately 150.6, and a half-life of at least 10 hours at 55 °C and/or a half life of at least 6.5 hours at 60 °C. In addition, the host is a food grade microorganism. These attributes are important in the bioproduction of a food-grade D-psicose with a more efficient production cycle and lower production costs.

Materials and Methods

Chemicals and reagents

[0072] Taq DNA polymerase, deoxynucleoside triphosphate (dNTP), chemicals for PCR, T4 DNA ligase and plasmid miniprep kit were obtained from Takara (Dalian, China). The resin for protein purification, the Chelating Sepharose Fast Flow, was obtained from GE (Uppsala, Sweden). Electrophoresis reagents were purchased from Bio-Rad. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and all chemicals used for enzyme assays and characterization were at least of analytical grade obtained from Sigma (St Louis, MO, USA) and Sinopharm Chemical Reagent (Shanghai, China). Oligonucleotides were synthesized by Sangon Biological Engineering Technology and Services (Shanghai, China).

Plamids, Bacterial strains, and Culture conditions

[0073] The plasmid pET-22b(+) was obtained from Novagen (Darmstadt, Germany). The *E. coli* DH5α and *E. coli* BL21(DE3) were obtained from Tiangen Biotechnology (Beijing, China). *Bacillus subtilis* WB600 and the plasmid pMA5 were obtained from Invitrogen (Carlsbad, CA, USA). The bacterial strains were grown in Luria-Bertani medium in a rotary shaker (200 rpm) at 37 °C.

Preparation of DPEase variants of *C. cellulolyticum* in *E.coli*

[0074] (1) Primers design for protein modification was as following:

Forward mutagenic primers:

G211S Forward primer1 :CATTTACACACTAGCGAATGTAATCGT (SEQ ID No 12)
G211A Forward primer2 :CATTTACACACTGCCGAATGTAATCGT (SEQ ID No 13)
G211D Forward primer3 :CATTTACACACTGACGAATGTAATCGT (SEQ ID No 14)
G211R Forward primer4 :CATTTACACACTCGCGAATGTAATCGT (SEQ ID No 15)
G211 W Forward primer5 :CATTTACACACTTGGGAATGTAATCGT (SEQ ID No 16)
G211L Forward primer6 :CATTTACACACTCTCGAATGTAATCGT (SEQ ID No 17)
Reverse primer: 5'-AGTGTGTAAATGTCCCAAGTAAGAGCCCGC-3' (SEQ ID No 18)

(2) Amplify the plasmid using the above primers by PCR technique.
Template: pET-Cc-dpe
DNA polymerase: Pfu
PCR program: PCR amplification was perfomed by Pfu DNA polymerase for 20 cycles consisting of 94 °C for 30s, 60 °C for 30 s, and 72 °C for 5 min, followed by extension step of 10 min at 72 °C.
(3) After PCR, add lul *Dpn*I restriction enzyme (10U/μl) into 200 μl PCR product, and incubate at 37 °C for 4 h, to digest and eliminate the template DNA.
(4) The DNA was purified by Gel Extraction Kit.
(5) The 5'-phosphorylation and ligation reactions of mutation fragments were performed together at 16 °C for 12h, and the reaction system was as follows:

Mutation DNA	7.5 μl
10×T4 ligase buffer	1 μl
PNK	0.5 μl
T4 ligase	1 μl

(6) The DNA was transformed into *E. coli* DH5α. The transformants were selected at 37°C on the LB agar plates containing 100 μg/mL ampicillin.
(7) The plasmid was extracted and identified by nucleotide sequencing.
(8) The reconstructed plasmid was transformed in to *E. coli* BL21
[0075] The transformants were selected at 37°C on the LB agar plates containing 100 μg/mL ampicillin.

Preparation of DPEase variants of *C. cellulolyticum* in *B. subtilis*

[0076]

(1) PCR
As to subclone the different variant genes to *B. subtilis* expression plasmid, forward (5'-CGCCATATGAAACATGG-TATATACTACGC-3' - SEQ ID No 19) and reverse primer (5'-CGCGGATCCTTGTTAGCCGGATCTC-3' - SEQ ID No 20) were designed to introduce the *Nde*I and *Bam*HI restriction sites. Using the reconstructed pET-22b(+) plasmids harboring different DPEase variant genes, PCR amplification were seperately performed by Taq Plus DNA polymerase for 35 cycles consisting of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, followed by a final extension step of 10 min at 72 °C.
(2) Purify the PCR products seperately using the Gel Extraction Kit.
(3) The purified PCR products and *B. subtilis* expression plasmid, pMA5 were digested by restriction enzyme *Nde*I

and *Bam*HI

(4) DNA fragment and pMA5 were ligated by T4 DNA Ligase, and then the mixture was transformed in to *E. coli* DH5α.

(5) The transformants were selected at 37 °C on the LB agar plates containing 100 μg/mL Ampicillin.

(6) The reconstructed plasmids were extracted and identified by restriction enzyme digestion and nucleotide sequencing.

(7) The reconstructed pMA5 plasmids harboring the wild-type or variant DPEase gene were seperately transformed in to *B. subtilis* WB600 by electroporation. The transformants were selected at 37°C on the LB agar plates containing 100 μg/mL Kanamycin.

Purification of DPEase variants of *C. cellulolyticum*

[0077] To purify the recombinant DPEase variants, the centrifuged cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) and disrupted by sonication at 4 °C for 6 min (pulsations of 3 s, amplify 90) using a Vibra-Cell 72405 sonicator, and cell debris was removed by centrifugation (20000g, 20 min, 4 °C). The cell-free extract was applied onto a Chelating Sepharose Fast Flow resin column (1.0 cm x 10.0 cm), previously chelating Ni^{2+}, and equilibrated with a binding buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5). Unbound proteins were eluted from the column with a washing buffer (50 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, pH 7.5). Then, the DPEase variants were eluted from the column with an elution buffer (50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.5). The active fractions were pooled and dialyzed overnight against 50mMTris-HCl buffer (pH 7.5) containing 10 mM ethylenediaminetetraacetic acid (EDTA) for 48 h at 4 °C. Subsequently, the enzyme was dialyzed against 50 mM EDTA-free Tris-HCl buffer (pH 7.5).

DPEase Assay

[0078] The activity was measured by the determination of the amount of produced D-psicose from D-fructose. The reaction mixture of 1 mL contained D-fructose (50 g/L), Tris-HCl buffer (50 mM, pH 8.0), 0.1mM Co^{2+}, and 0.5 μM enzyme. The reaction mixture was incubated at 55 °C for 2 min, and the reaction was stopped after 10 min by boiling. The generated D-psicose was determined by the HPLC method. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of D-psicose/min at pH 8.0 and 55 °C.

Effect of Temperature and pH

[0079] The optimum temperature of enzyme activity was measured by assaying the enzyme samples over the range of 35-70 °C for 2 min. Two buffer systems, sodium phosphate (50 mM, pH 6.0-7.0) and Tris-HCl (50 mM, pH 7.5-9.0), were used for measuring the optimum pH of enzyme activity. The thermal stability of the enzyme was studied by incubating the enzyme in Tris-HCl buffer (50 mM, pH 8.0) at various temperatures. At given time intervals, samples were withdrawn and the residual activity was measured under standard assay conditions. To determine the pH stability, the enzyme was incubated at pH 6.0-9.0 at 4 °C for up to 2 h, and the remaining enzyme activity was measured at time intervals under standard assay conditions.

Determination of kinetic parameters

[0080] Kinetic parameters of DPEase variants were determined in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1mM Co^{2+} and 5-200mM substrate for reaction at 55 °C. The enzyme reactions were stopped after 10 min by boiling, and the amount of D-psicose was determined by the HPLC assay. Kinetic parameters, such as the Michaelis-Menten constant (K_m) and turnover number (k_{cat}) values for substrates, were obtained using the Lineweaver-Burk equation and quantification of enzyme concentration.

Analytical Methods

[0081] The concentrations of D-fructose and D-psicose were analyzed by HPLC equipped with a refractive index detector and a Ca^{2+}-carbohydrate column (Waters Sugar-Pak 1, Waters Corp., Milford, MA), which was eluted with water at 85 °C and 0.4 mL/min. Protein concentration was determined according to the method of Bradford using bovine serum albumin as a standard. SDS-PAGE was carried out according to the method of Laemmli. Gels (12% w/v polyacrylamide) were stained with Coomassie Brilliant Blue and destained with an aqueous mixture of 10% (v/v) methanol/10% (v/v) acetic acid.

Table 1: Enzyme properties and kinetic parameters of the wild-type and mutant enzymes of DPEase from C. cellulolyticum for substrate D-psicose

Enzyme	**Optimum pH**	Optimum temp. (°C)	Equilibrium ratio between D-psicose and D-fructose at 55 °C	**Half-life at 60 °C (h)**	**k_{cat}/K_m for D-fructose (mM^{-1} min^{-1})**
Wild type	**8.0**	55	32:68	**6.8**	**62.7**
G211S	**6.5**	55	33:67	**7.2**	**150.6**
G211A	**8.5**	50	30:70	**2.5**	**9.8**
G211D	**8.0**	60	32:68	**5.3**	**86.7**
G211R	**6.5**	45	28:72	**3.7**	**26.5**
G211W	**7.0**	60	32 :68	**5.9**	**68.1**
G211L	**8.0**	55	30 :70	**6.7**	**73.2**
[a] NR, not reported.					

Table 2: Enzyme properties and kinetic parameters of DTEase family enzymes for D-psicose production

DTEase family enzymes	**Optimum pH**	Optimum temp. (°C)	Equilibrium ratio between D-psicose and D-fructose	**Half-life (thermostability)**	**k_{cat}/K_m for D-fructose (mM^{-1} min^{-1})**	Reference
C. cellulolyticum DPEase mutant of G211 S	**6.5**	55	33:67 (55 °C)	**10.1 h (55°C)** **7.2 h (60 °C)**	**150.6**	Invention
C. cellulolyticum DPEase	**8.0**	55	32:68 (55 °C)	**9.5 h (55 °C)** **6.8 h (60 °C)**	**62.7**	Mu et al. 2011
Clostridium sp. DPEase	**8.0**	65	28:72 (65 °C)	**0.25 h[b] (60 °C)**	**58.7**	Mu et al. 2013
C. bolteae	**7.0**	55	32:68 (60 °C)	**2.6 h[b] (55 °C)**	**59.4[c]**	Jia et al. 2013
C. scindens	**7.5**	60	28:72 (50 °C)	**1.8 h[b] (50 °C)**	**8.72**	Zhang et al. 2013
Ruminococcus sp. DPEase	**7.5-8.0**	60	28:72	**1.6 h (60 °C)**	**16**	Zhu et al. 2012
A. tumefaciens DPEase	**8.0**	50	32:68 (30 °C) 33:67 (40 °C)	**8.90 min (55 °C)** **3.99 min (60 °C)**	**85**	Kim et al. 2006
A. tumefaciens DPEase mutant of S213C	**NR[a]**	NR	NR	**0.46 h (55 °C)**	**101**	Choi et al. 2011
A. tumefaciens DPEase mutant of I33L	NR	NR	NR	**1.06 h (55 °C)**	**105**	Choi et al. 2011
A. tumefaciens DPEase mutant of S213C+I33L	**NR**	NR	NR	**4.4 h (55 °C)**	**134**	Choi et al. 2011
Rhizobium DTEase	**9.0-9.5**	50	23:77	**NR**	**NR**	Maruta et al. 2010
P. cichorii DTEase	**7.5**	60	20:80 (30 °C)	**1 h (50 °C)**	**NR**	Itoh et al. 1994

(continued)

DTEase family enzymes	**Optimum pH**	Optimum temp. (°C)	Equilibrium ratio between D-psicose and D-fructose	**Half-life (thermostability)**	**k_{cat}/K_m for D-fructose (mM^{-1} min^{-1})**	Reference
R. sphaeroides DTEase	**9.0**	40	23:77 (40 °C)	**NR**	**NR**	Zhang et al. 2009

[a] NR, not reported.
[b] The half-life values were converted from the original references with the unit of min.
[a] The value was converted from the orginal reference with the unit of mM^{-1} s^{-1}.

SEQUENCE LISTING TABLE

SEQ ID No	Description
1	Nucleic acid sequence of the parent D-psicose 3-epimerase from *Clostridium cellulolyticum*
2	Amino acid sequence of the parent D-psicose 3-epimerase from *Clostridium cellulolyticum*
3	Nucleic acid sequence of the D-psicose 3-epimerase variant derived from *Clostridium cellulolyticum*
4	Amino acid sequence of the D-psicose 3-epimerase variant derived from *Clostridium cellulolyticum*
5	Amino acid sequence of *Clostridium sp.* DPEase
6	Amino acid sequence of *C. scindens* DPEase
7	Amino acid sequence of *A. tumefaciens* DPEase
8	Amino acid sequence of *Ruminococcus* sp. DPEase
9	Amino acid sequence of *C. bolteae* DPEase
10	Amino acid sequence of *P. cichorii* DTEase
11	Amino acid sequence of *R. sphaeroides* DTEase
12-20	Primers

SEQUENCE LISTING

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Trp
                                100                 105                 110

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Arg
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100 105 110

Pro Val Asp Tyr Thr Lys Thr Ile Asp Lys Lys Gly Asp Trp Glu Arg
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Ser Val Glu Ser Val Arg Glu Val Ala Gln Val Ala Glu Ala Cys Gly
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Val Asp Phe Cys Leu Glu Val Leu Asn Arg Phe Glu Asn Tyr Leu Ile
145 150 155 160

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165 170 175

Asn Val Lys Val Met Leu Asp Thr Phe His Met Asn Ile Glu Glu Asp
180 185 190

Ser Ile Gly Gly Ala Ile Arg Thr Ala Gly Ser Tyr Leu Gly His Leu

195 200 205

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210 215 220

Trp Ile Glu Ile Gly Glu Ala Leu Ala Asp Ile Gly Tyr Asn Gly Ser
225 230 235 240

Val Val Met Glu Pro Phe Val Arg Met Gly Gly Thr Val Gly Ser Asn
245 250 255

Ile Lys Val Trp Arg Asp Ile Ser Asn Gly Ala Asp Glu Glu Lys Leu
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Asn Arg Lys Leu
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35 40 45

Lys Glu Leu Lys Lys Cys Ala Asp Asp Asn Gly Ile Gln Leu Thr Ala
50 55 60

Gly Tyr Gly Pro Ala Phe Asn His Asn Met Gly Ser Ser Asp Pro Lys
65 70 75 80

Ile Arg Glu Glu Ala Leu Gln Trp Tyr Lys Arg Leu Phe Glu Val Met
85 90 95

Ala Gly Leu Asp Ile His Leu Ile Gly Gly Ala Leu Tyr Ser Tyr Trp
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Pro Val Asp Phe Ala Thr Ala Asn Lys Glu Glu Asp Trp Lys His Ser
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Asn Leu Gly Met Glu Val Leu Asn Arg Phe Glu Ser His Ile Leu Asn
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Thr Ser Glu Glu Gly Val Lys Phe Val Thr Glu Val Gly Met Asp Asn
165 170 175

Val Lys Val Met Leu Asp Thr Phe His Met Asn Ile Glu Glu Ser Ser
180 185 190

Ile Gly Asp Ala Ile Arg His Ala Gly Lys Leu Leu Gly His Phe His
195 200 205

Thr Gly Glu Cys Asn Arg Met Val Pro Gly Lys Gly Arg Thr Pro Trp
210 215 220

Arg Glu Ile Gly Asp Ala Leu Arg Glu Ile Glu Tyr Asp Gly Thr Val
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Val Met Glu Pro Phe Val Arg Met Gly Gly Gln Val Gly Ser Asp Ile
245 250 255

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35 40 45

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65 70 75 80

Val Arg Ala Ala Gly Lys Ala Phe Phe Glu Arg Thr Leu Ser Asn
Val
85 90 95

Ala Lys Leu Asp Ile His Thr Ile Gly Gly Ala Leu His Ser Tyr
Trp
100 105 110

Pro Ile Asp Tyr Ser Gln Pro Val Asp Lys Ala Gly Asp Tyr Ala
Arg
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Gly Val Glu Gly Ile Asn Gly Ile Ala Asp Phe Ala Asn Asp Leu
Gly
130 135 140

Ile Asn Leu Cys Ile Glu Val Leu Asn Arg Phe Glu Asn His Val
Leu
145 150 155
160

Asn Thr Ala Ala Glu Gly Val Ala Phe Val Lys Asp Val Gly Lys
Asn
165 170 175

Asn Val Lys Val Met Leu Asp Thr Phe His Met Asn Ile Glu Glu
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180 185 190

Ser Phe Gly Asp Ala Ile Arg Thr Ala Gly Pro Leu Leu Gly His
Phe
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Trp His Glu Ile Gly Leu Ala Leu Arg Asp Ile Asn Tyr Thr Gly
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225 230 235

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Leu
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Tyr Trp Pro Val Asp Tyr Ser Lys Pro Phe Asp Lys Lys Arg Asp Leu
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35 40 45

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Thr Lys Ala Glu Asn Leu Cys Ser Glu Asp Pro Glu Ala Val Arg Arg
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Leu Arg Glu Leu Ser Lys Thr Ala Glu Glu Cys Asp Val Val Leu Gly
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180 185 190

Met Leu Asp Thr Phe His Met Asn Ile Glu Glu Thr Asn Met Ala Asp
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225 230 235 240

Gly Gln Ala Leu Arg Asp Ile Asn Tyr Gln Gly Ala Ala Val Met Glu
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Pro Phe Val Met Gln Gly Gly Thr Ile Gly Ser Glu Ile Lys Val Trp
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Lys
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Lys Arg Glu Leu Lys Ala Val Ala Asp Asp Leu Gly Leu Thr Val
Met
50 55 60

Cys Cys Ile Gly Leu Lys Ser Glu Tyr Asp Phe Ala Ser Pro Asp
Lys
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Ser Val Arg Asp Ala Gly Thr Glu Tyr Val Lys Arg Leu Leu Asp
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Cys His Leu Leu Gly Ala Pro Val Phe Ala Gly Leu Thr Phe Cys
Ala
100 105 110

Trp Pro Gln Ser Pro Pro Leu Asp Met Lys Asp Lys Arg Pro Tyr
Val
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Tyr Gly Ile Ile Tyr Ala Leu Glu Val Val Asn Arg Phe Glu Gln Trp
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Leu Cys Asn Asp Ala Lys Glu Ala Ile Ala Phe Ala Asp Ala Val Asp
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Ser Pro Ala Cys Lys Val Gln Leu Asp Thr Phe His Met Asn Ile Glu
180 185 190

Glu Thr Ser Phe Arg Asp Ala Ile Leu Ala Cys Lys Gly Lys Met Gly
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His Phe His Leu Gly Glu Ala Asn Arg Leu Pro Pro Gly Glu Gly Arg
210 215 220

Leu Pro Trp Asp Glu Ile Phe Gly Ala Leu Lys Glu Ile Gly Tyr Asp
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Gly Thr Ile Val Met Glu Pro Phe Met Arg Lys Gly Gly Ser Val Ser
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195 200 205

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Gly Thr Gly Thr Met Asp Trp Thr Ala Ile Ala Arg Ala Leu Gly
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240

Ala Gly Tyr Ala Gly Pro Val Ser Leu Glu Pro Phe Arg Arg Asp
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25

Claims

1. A variant of a parent D-psicose 3-epimerase, wherein the variant comprises a substitution of a Glycine residue by a Serine residue at a position corresponding to the position 211 in SEQ ID No 2 compared to the parent D-psicose 3-epimerase; and wherein the variant has a D-psicose 3-epimerase activity.

2. The variant according to claim 1, wherein the variant has one or several following features:

   a. a lower pH optimum compared to the parent D-psicose 3-epimerase, preferably in the range of 6 to 7; and/or
   b. a higher catalysis efficiency to the substrate D-fructose compared to the parent-psicose 3-epimerase, preferably at least twice higher; and/or
   c. a longer half-life at 60°C compared to the parent-psicose 3-epimerase.

3. The variant according to claim 1 or 2, wherein the variant has an amino acid sequence having 35 % of identity or higher with SEQ ID No 2, preferably 60 % of identity or higher, more preferably at least 70, 75, 80, 85, 90, 95 % of identity or higher.

4. The variant according to any one of claims 1-3, wherein the parent D-psicose 3-epimerase is selected from a D-tagatose 3-epimerase from *Pseudomonas cichorii,* a D-psicose 3-epimerase from *Agrobacterium tumefaciens,* a D-psicose 3-epimerase from *Clostridium sp,* a D-psicose 3-epimerase from *Clostridium scindens,* a D-psicose 3-epimerase from *Clostridium bolteae,* a D-psicose 3-epimerase from *Ruminococcus sp,* and a D-psicose 3-epimerase from *Clostridium cellulolyticum.*

5. The variant according to any one of claims 1-4, wherein the parent D-psicose 3-epimerase is the D-psicose 3-epimerase from *Clostridium cellulolyticum.*

6. The variant according to claim 5, wherein the variant comprises or consists of the amino acid sequence of SEQ ID No 4 or an amino acid sequence having 90 or 95 % of identity with SEQ ID No 4 or higher with a residue Ser at position 211.

7. An isolated nucleic acid encoding a variant according to any one of claims 1-6.

8. A recombinant expression vector comprising a nucleic acid according to claim 7.

9. A recombinant host cell comprising a nucleic acid according to claim 7 or a recombinant expression vector according to claim 8.

10. The recombinant host cell according to claim 9, wherein the host cell is a GRAS strain (Generally Recognized As Safe), preferably *Bacillus subtilis.*

11. A method for producing a D-psicose 3-epimerase variant comprising culturing the recombinant host cell according to claim 9 or 10, and optionally recovering the produced D-psicose 3-epimerase variant from the resulting culture.

12. A method for producing D-psicose comprising contacting a variant according to any one of claims 1 to 6 with D-fructose in conditions suitable for the D-psicose 3-epimerase activity and optionally recovering the produced D-psicose.

13. The method of claim 12, wherein the D-fructose is previously or simultaneously produced by a glucose isomerase from D-glucose.

14. An enzymatic composition comprising a D-psicose 3-epimerase variant according to any one of claims 1 to 6 and an additional enzyme, in particular a glucose isomerase.

15. Use of a D-psicose 3-epimerase variant according to any one of claims 1 to 6 or a host cell according to claim 9 or 10 for producing D-psicose.

16. Use of a host cell according to claim 10 for preparing a food product.

17. A food product comprising a host cell according to claim 10.

```
Clce-DPEase      ----------MKHGIYYAYWEQEWEADYKYYIEKVAKLGFDILEIAASPLPFYS--DIQI 48
Clsp-DPEase      ----------MKHGIYYAYWEQEWEADYKYYIEKVAKLGFDILEIAASPLPFYS--DNQI 48
Clsc-DPEase      ----------MKHGIYYAYWEQEWAADYKRYVEKAAKLGFDILEVGAAPLPDYS--AQEV 48
Agtu-DPEase      ----------MKHGIYYSYWEHEWSAKFGPYIEKVAKLGFDIIEVAAHHINEYS--DAEL 48
Rusp-DPEase      ----------MKYGIYYAYWEKEWNGDYKYYIDKISKLGFDILEISCGAFSDYYTKDQEL 50
Clbo-DPEase      MRYFKEEVAGMKYGIYFAYWTKEWFADYKKYMDKVSALGFDVLEISCAALRDVYTTKEQL 60
Psci-DTEase      ---------MNKVGMFYTYWSTEWMVDFPATAKRIAGLGFDLMEISLGEFHNLS--DAKK 49
Rhsp-DTEase      --------MKNPVGIISMQFIRPFTSESLHFLKKSRALGFDFIELLVPEPEDGL----DA 48
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Clsp-DPEase      NELKACARGNGITLTVGHGPSAEQNLSSPDPYIRKNAKAFYTDLLKRLYKLDVHLIGGAI 108
Clsc-DPEase      KELKKCADDNGIQLTAGYGPAFNHNMGSSDPKIREEALQWYKRLFEVMAGLDIHLIGGAL 108
Agtu-DPEase      ATIRKSAKDNGIILTAGIGPSKTKNLSSEDAAVRAAGKAFFERTLSNVAKLDIHTIGGAL 108
Rusp-DPEase      IDIGKYAKEKGVTLTAGYGPHFNESLSSSEPNTQKQAISFWKETLRKLKLMDIHIVGGAL 110
Clbo-DPEase      IELREYAKEKGLVLTAGYGPTKAENLCSEDPEAVRRAMTFFKDLLPKLQLMDIHILGGGL 120
Psci-DTEase      RELKAVADDLGLTVMCCIGLKSEYDFASPDKSVRDAGTEYVKRLLDDCHLLGAPVFAGLT 109
Rhsp-DTEase      AEVRRICEGEGLGLVLAARVNLQRSIASEEAAARAGGRDYLKYCIEAAEALGATIVGGPL 108
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Clsp-DPEase      YSY-----WPVDYTKTIDKKGDW-ERSVESVREVAQVAEACGVDFCLEVLNRFENYLINT 162
Clsc-DPEase      YSY-----WPVDFA-TANKEEDW-KHSVEGMQILAPIASQYGINLGMEVLNRFESHILNT 161
Agtu-DPEase      HSY-----WPIDYSQPVDKAGDY-ARGVEGINGIADFANDLGINLCIEVLNRFENHVLNT 162
Rusp-DPEase      YGY-----WPVDYSKPFDKKRDL-ENSIKNMKIISQYAEEYDIMMGMEVLNRFEGYMLNT 164
Clbo-DPEase      YSY-----WPVDFTINNDKQGDR-ARAVRNLRELSKTAEECDVVLGMEVLNRYEGYILNT 174
Psci-DTEase      FCA-----WPQSPPLDMKDKRPYVDRAIESVRRVIKVAEDYGIIYALEVVNRFEQWLCND 164
Rhsp-DTEase      YGEPLVFAGRPPFPWTAEQIATRAARTVEGLAEVAPLAASAGKVFGLEPLNRFETDIVNT 168
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Clsc-DPEase      SEEGVKFVTEVGMDNVKVMLDTFHMNIEESSIGDAIRHAGKLLGHFHTGECNRMVPGKGR 221
Agtu-DPEase      AAEGVAFVKDVGKNNVKVMLDTFHMNIEEDSFGDAIRTAGPLLGHFHTGESNRRVPGKGR 222
Rusp-DPEase      CDEALAYVEEVGSSNVGVMLDTFHMNIEEDNIAAAIRKAGDRLVHFHIGEGNRKVPGKGM 224
Clbo-DPEase      CEEAIDFVDEIGSSHVKIMLDTFHMNIEETNMADAIRKAGDRLGHLHLGEQNRLVPGKGS 234
Psci-DTEase      AKEAIAFADAVDSPACKVQLDTFHMNIEETSFRDAILACKGKMGHFHLGEANRLPPGEGR 224
Rhsp-DTEase      TAQAIEVVDAVGSPGLGVMLDTFHMNMEERSIPDAIRATGARLVHFQANENHRGFPGTGT 228
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Clsp-DPEase      IPWIEIGEALADIGYNGSVVMEPFVRMGGTVGSNIKVWRDISNGADEEKLDREAQAALNF 282
Clsc-DPEase      TPWREIGDALREIEYDGTVVMEPFVRMGGQVGSDIKVWRDISKGAGEDRLDEDARRAVEF 281
Agtu-DPEase      MPWHEIGLALRDINYTGAVIMEPFVKTGGTIGSDIKVWRDLSGGADIAKMDEDARNALAF 282
Rusp-DPEase      LPWNEIGQALRDINYQHAAVMEPFVMQGGTVGHDIKIWRDIIGNCSEVTLDMDAQSALHF 284
Clbo-DPEase      LPWAEIGQALRDINYQGAAVMEPFVMQGGTIGSEIKVWRDMVPDLSEEALDRDAKGALEF 294
Psci-DTEase      LPWDEIFGALKEIGYDGTIVMEPFMRKGGSVSRAVGVWRDMSNGATDEEMDERARRSLQF 284
Rhsp-DTEase      MDWTAIARALGQAGYAGPVSLEPFRRDDERVALPIAHWR-----APHEDEDEKLRAGLGL 283
                   *  *  ** :  *  .  :***   .  :.  :  **          *   : .: :

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Clsp-DPEase      SRYVLGNRKL-- 292
Clsc-DPEase      QRYMLEWK---- 289
Agtu-DPEase      SRFVLGG----- 289
Rusp-DPEase      VKHVFEV----- 291
Clbo-DPEase      CRHVFGI----- 301
Psci-DTEase      VRDKLA------ 290
Rhsp-DTEase      IRSAITLAEVTH 295
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FIGURE 1

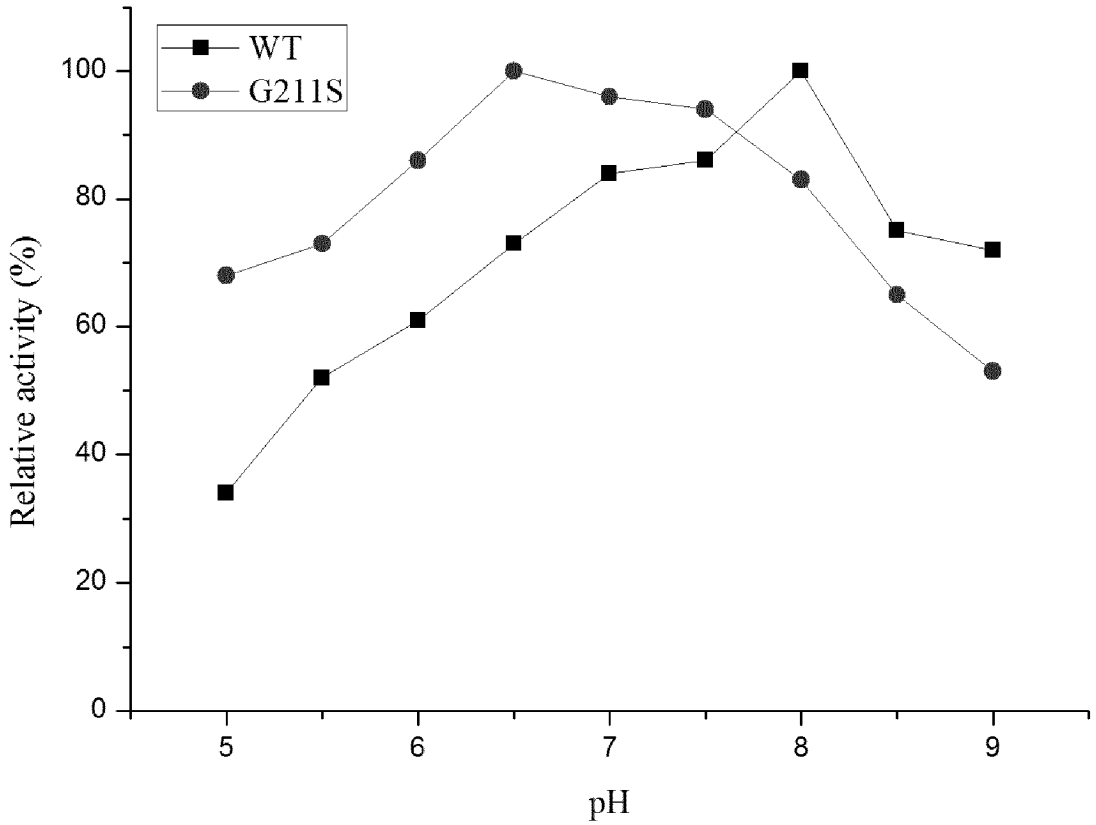

FIGURE 2

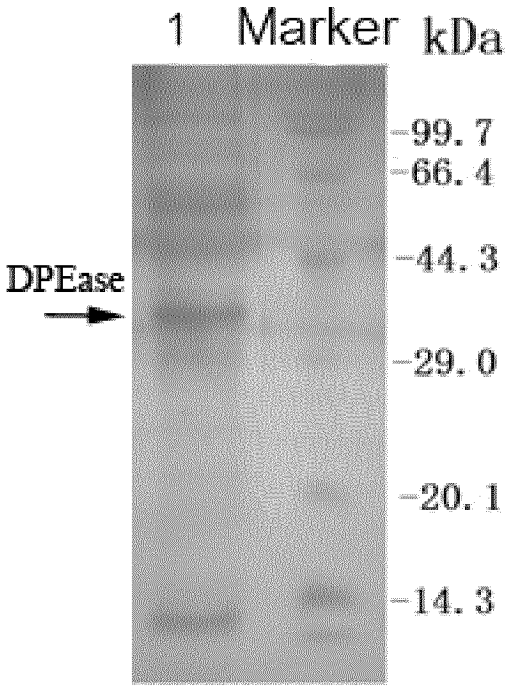

FIGURE 3

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 13 30 6195

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	DATABASE UniProt [Online] 23 March 2010 (2010-03-23), "SubName: Full=AP endonuclease, family 2;", XP002716904, retrieved from EBI accession no. UNIPROT:D3AJP4 Database accession no. D3AJP4 * sequence * -----	1,3-5	INV. C12N9/90 A23L1/09
A	J.-G. CHOI ET AL: "Improvement in the Thermostability of D-Psicose 3-Epimerase from Agrobacterium tumefaciens by Random and Site-Directed Mutagenesis", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 77, no. 20, 15 October 2011 (2011-10-15), pages 7316-7320, XP055089545, ISSN: 0099-2240, DOI: 10.1128/AEM.05566-11 * the whole document * -----	1-17	TECHNICAL FIELDS SEARCHED (IPC)
A	WO 2013/027999 A2 (CJ CHEILJEDANG CORP [KR]; KIM YANG HEE [KR]; KIM JIN HA [KR]; LEE YOUN) 28 February 2013 (2013-02-28) * the whole document * -----	1-17	C12N A23L
A,D	WO 2011/040708 A2 (CJ CHEILJEDANG CORP [KR]; HONG YOUNG HO [KR]; KIM JIN HA [KR]; KIM SUN) 7 April 2011 (2011-04-07) * claims * -----	1-17	
	-/--		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	25 November 2013	Huber, Angelika

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 13 30 6195

DOCUMENTS CONSIDERED TO BE RELEVANT

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	25 November 2013	Huber, Angelika

CATEGORY OF CITED DOCUMENTS

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EUROPEAN SEARCH REPORT

Application Number
EP 13 30 6195

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